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(54) Title: FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF

(57) Abstract

The present invention is directed to novel fluorescent proteins from non-bioluminescent organisms from the Class Anthozoa. Also disclosed are cDNAs encoding the fluorescent proteins.

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FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF

BACKGROUND OF THE INVENTION

Cross-reference to Related Application

This is a divisional application of U.S.S.N. 09/210,330 filed on December 11, 1998.

Field of the Invention

This invention relates to the field of molecular biology. More specifically, this invention relates to novel fluorescent proteins, cDNAs encoding the proteins and uses thereof.

Description of the Related Art

Fluorescence labeling is a particularly useful tool for marking a protein, cell, or organism of interest. Traditionally, a protein of interest is purified, then covalently conjugated to a fluorophore derivative. For *in vivo* studies, the protein-dye complex is then inserted into cells of interest using micropipetting or a method of reversible permeabilization. The dye attachment and insertion steps, however, make the process laborious and difficult to control. An alternative method of labeling proteins of interest is to concatenate or fuse the gene expressing the protein of interest to a gene expressing a marker, then express the fusion product. Typical markers for this

method of protein labeling include β-galactosidase, firefly luciferase and bacterial luciferase. These markers, however, require exogenous substrates or cofactors and are therefore of limited use for *in vivo* studies.

A marker that does not require an exogenous cofactor or substrate is the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*, a protein with an excitation maximum at 395 nm, a second excitation peak at 475 nm and an emission maximum at 510 nm. GFP is a 238-amino acid protein, with amino acids 65-67 involved in the formation of the chromophore.

Uses of GFP for the study of gene expression and protein localization are discussed in detail by Chalfie et al. in *Science* 263 (1994), 802-805, and Heim et al. in *Proc. Nat. Acad. Sci.* 91 (1994), 12501-12504. Additionally, Rizzuto et al. in *Curr. Biology* 5 (1995), 635-642, discuss the use of wild-type GFP as a tool for visualizing subcellular organelles in cells, while Kaether and Gerdes in *Febs Letters* 369 (1995), 267-271, report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng in *Febs Letters* 369 (1995), 331-334, while GFP expression in *Drosophila* embryos is described by Davis et al. in *Dev. Biology* 170 (1995), 726-729.

Crystallographic structures of wild-type GFP and the mutant GFP S65T reveal that the GFP tertiary structure resembles a barrel (Ormö et al., *Science* 273 (1996), 1392-1395; Yang, et al., *Nature Biotechnol* 14 (1996), 1246-1251). The barrel consists of beta sheets in a compact structure, where, in the center, an alpha helix containing the chromophore is shielded by the barrel. The compact structure makes GFP very stable under diverse and/or harsh conditions such as protease treatment, making GFP an extremely useful reporter in

general. However, the stability of GFP makes it sub-optimal for determining short-term or repetitive events.

A great deal of research is being performed to improve the properties of GFP and to produce GFP reagents useful and optimized for a variety of research purposes. New versions of GFP have been developed, such as a "humanized" GFP DNA, the protein product of which has increased synthesis in mammalian cells (Haas, et al., *Current Biology* 6 (1996), 315-324; Yang, et al., *Nucleic Acids Research* 24 (1996), 4592-4593). One such humanized protein is "enhanced green fluorescent protein" (EGFP). Other mutations to GFP have resulted in blue-, cyan- and yellow-green light emitting versions. Despite the great utility of—GFP, however, other fluorescent proteins with properties similar to or different from GFP would be useful in the art. Novel fluorescent proteins result in possible new colors, or produce pH-dependent fluorescence. Other benefits of novel fluorescent proteins include fluorescence resonance energy transfer (FRET) possibilities based on new spectra and better suitability for larger excitation.

The prior art is deficient in novel fluorescent proteins wherein the DNA coding sequences are known. The present invention fulfills this long-standing need in the art.

SUMMARY OF THE INVENTION

The present invention is directed to DNA sequences encoding fluorescent proteins selected from the group consisting of: (a) an isolated DNA from an organism from the Class Anthozoa which encodes a fluorescent protein; (b) an isolated DNA which hybridizes to the isolated DNA of (a) and which encodes a fluorescent protein; and (c) an isolated DNA differing from the isolated DNAs of (a) and (b) in

codon sequence due to the degeneracy of the genetic code and that encodes a fluorescent protein. Preferably, the DNA is isolated from a non-bioluminescent organism from Class Anthozoa. More preferably, the DNA has the sequence selected from the group consisting of SEQ ID Nos. 55, 57 and 59; and the fluorescent protein has the amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58.

In another embodiment of the present invention, there is provided a vector capable of expressing the DNA of the present invention in a recombinant cell comprising said DNA and regulatory elements necessary for expression of the DNA in the cell. Preferably, the DNA encodes a fluorescent protein having the amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58.

In still another embodiment of the present invention, there is provided a host cell transfected with a vector of the present invention, such that the host cell expresses a fluorescent protein. Preferably, the cell is selected from the group consisting of bacterial cells, mammalian cells, plant cells, insect cells and yeast cells.

The present invention is also directed to an isolated and purified fluorescent protein coded for by DNA selected from the group consisting of: (a) isolated DNA from an organism from Class Anthozoa which encodes a fluorescent protein; (b) isolated DNA which hybridizes to the isolated DNA of (a) and which encodes a fluorescent protein; and (c) isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to the degeneracy of the genetic code, and which encodes a fluorescent protein. Preferably, the protein has the amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58.

The present invention is also directed to a DNA sequence encoding a fluorescent protein selected from the group consisting of: (a) an isolated DNA which encodes a fluorescent protein, wherein said

DNA is from an organism from Class Anthozoa and wherein said organism does not exhibit bioluminescence; (b) an isolated DNA which hybridizes to isolated DNA of (a) and which encodes a fluorescent protein; and (c) an isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein. Preferably, the organism is from Sub-class Zoantharia, Order Actiniaria. More preferably, the organism is from Sub-order Endomyaria. Even more preferably, the organism is from Family Actiniidae, Genus Anemonia. Even more preferably, the organism is *Anemonia sulcata*. Most particularly, the present invention is drawn to a novel fluorescent protein from *Anemonia sulcata*, asFP600 (wild type) and an engineered mutant of this novel fluorescent protein, Mut1.

The present invention is further directed to an amino acid sequence which can be used as a basis for designing an oligonucleotide probe for identification of a DNA encoding a fluorescent protein by means of hybridizaton, wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12, 14. Preferably, such an oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the modified strategy of 3'-RACE used to isolate the target fragments. Sequences of the oligonucleotides used

are shown in Table 2. Dp1 and Dp2 are the degenerate primers used in the first and second PCR, respectively (see Tables 3 and 4 for the sequences of degenerate primers). In the case of *Anemonia sulcata*, the first degenerate primer used was NGH (SEQ ID No. 4), and the second degenerate primer used was GEGa (SEQ ID No. 6) or NFP (SEQ ID No. 13).

Figure 2 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia sulcata*, asFP600.

Figure 3 shows the excitation and emission spectrum of Mut1.

Figure 4 shows that the expression of asFP600 concentrated at the nucleus. Non-humanized mutant asFP600 (RNFP) DNA were amplified via PCR and reconstructed to EGFP-N1 backbone. This vector (pRNFP-N1) was used for transient transfection in 293 cells. 24 hours post transfection, expression of asFP600 was examined under fluorescent microscope.

Figure 5 shows the transfection of nuclear exported asFP600 (NE-asFP600) in 293 cells. 24 hours post transfection, expression of NE-asFP600 was examined under fluorescence microscope. Red fluorescence was observed to be distributed in the cytosol but not in the nucleus.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "GFP" refers to the basic green fluorescent protein from *Aequorea victoria*, including prior art versions of GFP engineered to provide greater fluorescence or fluoresce in different colors. The sequence of *Aequorea victoria* GFP (SEQ ID No. 54) has been disclosed in Prasher et al., *Gene* 111 (1992), 229-33.

As used herein, the term "EGFP" refers to mutant variant of GFP having two amino acid substitutions: F64L and S65T (Heim et al., Nature 373 (1995), 663-664). The term "humanized" refers to changes made to the GFP nucleic acid sequence to optimize the codons for expression of the protein in human cells (Yang et al., *Nucleic Acids Research* 24 (1996), 4592-4593).

As used herein, the term "NFP" refers to novel fluorescent protein. "RNFP" refers to red novel fluorescent protein. Specifically, "RNFP" refers to asFP600.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells and Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes

double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. A polyadenylation signal and transcription termination sequence may be located 3' to the coding sequence.

As used herein, the term "hybridization" refers to the process of association of two nucleic acid strands to form an antiparallel duplex stabilized by means of hydrogen bonding between residues of the opposite nucleic acid strands.

The term "oligonucleotide" refers to a short (under 100 bases in length) nucleic acid molecule.

"DNA regulatory sequences", as used herein, are transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for and/or regulate expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above

background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the various vectors of the present invention.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example,

heterologous DNA includes coding sequence in a construct where portions of genes from two different sources have been brought together so as to produce a fusion protein product. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the term "reporter gene" refers to a coding sequence attached to heterologous promoter or enhancer elements and whose product may be assayed easily and quantifiably when the construct is introduced into tissues or cells.

The amino acids described herein are preferred to be in the "L" isomeric form. The amino acid sequences are given in one-letter code (A: alanine; C: cysteine; D: aspartic acid; E: gluetamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: metionine; N: asparagine; P: proline; Q: gluetamine; R: arginine; S: serine; T: threonine; V: valine; W: tryptophane; Y: tyrosine; X: any residue). NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243 (1969), 3552-59 is used.

The present invention is directed to an isolated DNA selected from the group consisting of: (a) isolated DNA from an organism from the Class Anthozoa which encodes a fluorescent protein; (b) isolated DNA which hybridizes to isolated DNA of (a) and which encodes a fluorescent protein; and (c) isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to the degeneracy of the genetic code, and which encodes a fluorescent protein.. Preferably, the DNA has the sequence selected from the group consisting of SEQ ID Nos. 55, 57 and 59 and the fluorescent protein has

the amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58. More preferably, the DNA is asFP600 or Mut1.

In another embodiment of the present invention, there is provided a vector capable of expressing the DNA of the present invention in a recombinant cell comprising said DNA and regulatory elements necessary for expression of the DNA in the cell. Specifically, the DNA encodes a fluorescent protein having the amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58. Preferably, the vector is constructed by amplifying the DNA and then inserting the amplified DNA into EGFP-N1 backbone, or by fusing different mouse ODC degradation domains such as d1, d2 and d376 to the C-terminal of the DNA and then inserting the fusion DNA into EGFP-N1 backbone.

In still another embodiment of the present invention, there is provided a host cell transfected with the vector of the present invention, which expresses a fluorescent protein of the present invention. Preferably, the cell is selected from the group consisting of bacterial cells, mammalian cells, plant cells, insect cells and yeast cells. A representative example of mammalian cell is HEK 293 cell and an example of bacterial cell is an *E. coli* cell.

The present invention is also directed to a DNA sequence encoding a fluorescent protein selected from the group consisting of: (a) an isolated DNA which encodes a fluorescent protein, wherein said DNA is from an organism from Class Anthozoa and wherein said organism does not exhibit bioluminescence; (b) an isolated DNA which hybridizes to isolated DNA of (a) and which encodes a fluorescent protein; and (c) an isolated DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein. Preferably, the organism is from Sub-class Zoantharia, Order Actiniaria. More preferably, the

organism is from Sub-order Endomyaria. Even more preferably, the organism is from Family Actiniidae, Genus Anemonia. Most preferably, the organism is *Anemonia sulcata.*

The present invention is also directed to an isolated and purified fluorescent protein coded for by DNA selected from the group consisting of: (a) an isolated protein encoded by a DNA which encodes a fluorescent protein wherein said DNA is from an organism from Class Anthozoa and wherein said organism does not exhibit bioluminescence; (b) an isolated protein encoded by a DNA which hybridizes to isolated DNA of (a); and (c) an isolated protein encoded by a DNA differing from the isolated DNAs of (a) and (b) in codon sequence due to degeneracy of the genetic code. Preferably, the isolated and purified fluorescent protein is asFP600.

The present invention is further directed to an amino acid sequence which can be used as a basis for designing an oligonucleotide probe for identification of a DNA encoding a fluorescent protein by means of hybridizaton, wherein the amino acid sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12, 14. Preferably, such an oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16 and is used as a primer in polymerase chain reaction. Alternatively, it can be used as a probe for hybridization screening of the cloned genomic or cDNA library.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Biological Material

Novel fluorescent proteins were identified from several genera of Anthozoa which do not exhibit any bioluminescence but have fluorescent color as observed under usual white light or ultraviolet light. Six species were chosen (see Table 1).

TABLE 1

Anthozoa Species Used in This Study

Species	Area of Origination	Fluorescent Color
Anemonia majano	Western Pacific	bright green tentacle tips
Clavularia sp.	Western Pacific	bright green tentacles and oral disk
Zoanthus sp.	Western Pacific	green-yellow tentacles and oral disk
Discosoma sp. "red"	Western Pacific	orange-red spots oral disk
Discosoma striata	Western Pacific	blue-green stripes on oral disk
Discosoma sp. "magenta"	Western Pacific	faintly purple oral disk
Discosoma sp. "green"	Western Pacific	green spots on oral disk
Anemonia sulcata	Mediterranean	purple tentacle tips

EXAMPLE 2

cDNA Preparation

Total RNA was isolated from the species of interest according to the protocol of Chomczynski and Sacchi (Chomczynski P., et al., *Anal. Biochem.* 162 (1987), 156-159). First-strand cDNA was synthetized starting with 1-3 μg of total RNA using SMART PCR cDNA synthesis kit (CLONTECH) according to the provided protocol with the only alteration being that the "cDNA synthesis primer" provided in the kit was replaced by the primer TN3 (5'- CGCAGTCGACCG$(T)_{13}$, SEQ ID No. 1) (Table 2). Amplified cDNA samples were then prepared as described in the protocol provided except the two primers used for PCR were the TS primer (5'-AAGCAGTGGTATCAACGCAGAGT, SEQ ID No. 2) (Table 2) and the TN3 primer (Table 2), both in 0.1 μM concentration. Twenty to twenty-five PCR cycles were performed to amplify a cDNA sample. The amplified cDNA was diluted 20-fold in water and 1 μl of this dilution was used in subsequent procedures.

TABLE 2

Oligos Used in cDNA Synthesis and RACE

TN3: 5'-CGCAGTCGACCG(T)$_{13}$
(SEQ ID No. 1)

T7-TN3: 5'-GTAATACGACTCACTATAGGGCCGCAGTCGACCG(T)$_{13}$
(SEQ ID No. 17)

TS-primer: 5'-AAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 2)

T7-TS:
5'-GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 18)

T7: 5'-GTAATACGACTCACTATAGGGC
(SEQ ID No. 19)

TS-oligo 5'-AAGCAGTGGTATCAACGCAGAGTACGCrGrGrG
(SEQ ID No. 53)

EXAMPLE 3

Oligo Design

To isolate fragments of novel fluorescent protein cDNAs, PCR using degenerate primers was performed. Degenerate primers were designed to match the sequence of the mRNAs in regions that were predicted to be the most invariant in the family of fluorescent proteins. Four such stretches were chosen (Table 3) and variants of degenerate primers were designed. All such primers were directed to the 3'-end of mRNA. All oligos were gel-purified before use. Table 2 shows the oligos used in cDNA synthesis and RACE.

TABLE 3

Key Amino Acid Stretches and Corresponding Degenerate Primers Used for Isolation of Fluorescent Proteins

Stretch Position according to A. victoria GFP (7)	Amino Acid Sequence of the Key Stretch	Degenerated Primer Name and Sequence
20-25	GXVNGH (SEQ ID No. 3)	NGH: 5'- GA(C,T) GGC TGC GT(A,T,G,C) AA(T,C) GG(A,T,G) CA (SEQ ID No. 4)
31-35	GEGEG (SEQ ID No. 5)	GEGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) GA(A,G) GG (SEQ ID No. 6) GEGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) GA(A,G) GG (SEQ ID No. 7)
	GEGNG (SEQ ID No. 8)	GNGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) AA(C,T) GG (SEQ ID No. 9) GNGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) AA(C,T) GG (SEQ ID No. 10)
127-131	GMNFP (SEQ ID No. 11) GVNFP (SEQ ID No. 12)	NFP: 5' TTC CA(C,T) GGT (G,A)TG AA(C,T) TT(C,T) CC (SEQ ID NO. 13)
134-137	GPVM (SEQ ID No. 14)	PVMa: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(A,C) ATG (SEQ ID NO. 15) PVMb: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(G,T) ATG (SEQ ID NO. 16)

EXAMPLE 4

Isolation of 3'-cDNA Fragments of nFPs

The modified strategy of 3'-RACE was used to isolate the target fragments (see Figure 1). The RACE strategy involved two consecutive PCR steps. The first PCR step involved a first degenerate primer (Table 4) and the T7-TN3 primer (SEQ ID No. 17) which has a 3' portion identical to the TN3 primer used for cDNA synthesis (for sequence of T7-TN3, Table 2). The reason for substituting the longer T7-TN3 primer in this PCR step was that background amplification which occurred when using the shorter TN3 primer was suppressed effectively, particularly when the T7-TN3 primer was used at a low concentration (0.1 _M) (Frohman et al., (1998) *PNAS USA*, 85, 8998-9002). The second PCR step involved the TN3 primer (SEQ ID No. 1, Table 2) and a second degenerate primer (Table 4).

TABLE 4

Combinations of Degenerate Primers for First and Second PCR Resulting in Specific Amplification of 3'-Fragments of nFP cDNA

Species	First Degenerate Primer	Second Degenerate Primer
Anemonia majano	NGH (SEQ ID No. 4)	GNGb (SEQ ID No. 10)
Clavularia sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Zoanthus sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Discosoma sp. "red"	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6), NFP (SEQ ID No. 13) or PVMb (SEQ ID No. 16)
Discosoma striata	NGH (SEQ ID No. 4)	NFP (SEQ ID No. 13)
Anemonia sulcata	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6) or NFP (SEQ ID No. 13)

The first PCR reaction was performed as follows: 1 µl of 20-fold dilution of the amplified cDNA sample was added into the reaction mixture containing 1X Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH), 200 µM dNTPs, 0.3 µM of first degenerate primer (Table 4) and 0.1 µM of T7-TN3 (SEQ ID No. 17) primer in a

total volume of 20 µl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C, 1 min.; 72°C, 40 sec; 24 cycles for 95°C, 10 sec.; 62°C, 30 sec.; 72°C, 40 sec. The reaction was then diluted 20-fold in water and 1 µl of this dilution was added to a second PCR reaction, which contained 1X Advantage KlenTaq Polymerase Mix with the buffer provided by the manufacturer (CLONTECH), 200 µM dNTPs, 0.3 µM of the second degenerate primer (Table 4) and 0.1 µM of TN3 primer. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C (for GEG/GNG or PVM) or 52°C (for NFP), 1 min.; 72°C, 40 sec; 13 cycles for 95°C, 10sec.; 62°C (for GEG/GNG or PVM) or 58°C (for NFP), 30 sec.; 72°C, 40 sec. The product of PCR was cloned into PCR-Script vector (Stratagene) according to the manufacturer's protocol.

Different combinations of degenerate primers were tried in the first and second PCR reactions on the DNA from each species until a combination of primers was found that resulted in specific amplification--meaning that a pronounced band of expected size (about 650-800 bp for NGH and GEG/GNG and 350-500 bp for NFP and PVM--sometimes accompanied by a few minor bands) was detected on agarose gel after two PCR reactions. The primer combinations of choice for different species of the Class Anthozoa are listed in Table 4. Some other primer combinations also resulted in amplification of fragments of correct size, but the sequence of these fragments showed no homology to the other fluorescent proteins identified or to *Aequorea victoria* GFP.

EXAMPLE 5

Obtaining Full-Length cDNA Copies

Upon sequencing the obtained 3'-fragments of novel fluorescent protein cDNAs, two nested 5'-directed primers were synthesized for cDNA (Table 5), and the 5' ends of the cDNAs were then amplified using two consecutive PCRs. In the next PCR reaction, the novel approach of "step-out PCR" was used to suppress background amplification. The step-out reaction mixture contained 1x Advantage KlenTaq Polymerase Mix using buffer provided by the manufacturer (CLONTECH), 200 µM dNTPs, 0.2 µM of the first gene-specific primer (see Table 5), 0.02 µM of the T7-TS primer (SEQ ID No. 18), 0.1 µM of T7 primer (SEQ ID No. 19) and 1 µl of the 20-fold dilution of the amplified cDNA sample in a total volume of 20 µl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of amplification was diluted 50-fold in water and one µl of this dilution was added to the second (nested) PCR. The reaction contained 1X Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH), 200 µM dNTPs, 0.2 µM of the second gene-specific primer and 0.1 µM of TS primer (SEQ ID No. 2) in a total volume of 20 µl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 12 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of amplification was then cloned into pAtlas vector (CLONTECH) according to the manufacturer's protocol.

TABLE 5

Gene-Specific Primers Used for 5'-RACE

Species	First Primer	Second (Nested) Primer
Anemonia majano	5'-GAAATAGTCAGGCATACTGGT (SEQ ID No. 20)	5'-GTCAGGCATAC TGGTAGGAT (SEQ ID No. 21)
Clavularia sp.	5'-CTTGAAATAGTCTGCTATATC (SEQ ID No. 22)	5'-TCTGCTATATC GTCTGGGT (SEQ ID No. 23)
Zoanthus sp.	5'-GTTCTTGAAATAGTCTACTATGT (SEQ ID No. 24)	5'-GTCTACTATGTCTT GAGGAT (SEQ ID No. 25)
Discosoma sp. "red"	5'-CAAGCAAATGGCAAAGGTC (SEQ ID No. 26)	5'-CGGTATTGTGGCC TTCGTA (SEQ ID No. 27)
Discosoma striata	5'-TTGTCTTCTTCTGCACAAC (SEQ ID No. 28)	5'-CTGCACAACGG GTCCAT (SEQ ID No. 29)
Anemonia sulcata	5'-CCTCTATCTTCATTTCCTGC (SEQ ID No. 30)	5'-TATCTTCATTTCCT GCGTAC (SEQ ID No. 31)
Discosoma sp. "magenta"	5'-TTCAGCACCCCATCACGAG (SEQ ID No. 32)	5'-ACGCTCAGAGCTG GGTTCC (SEQ ID No. 33)
Discosoma sp. "green"	5'-CCCTCAGCAATCCATCACGTTC (SEQ ID No. 34)	5'-ATTATCTCAGTGGA TGGTTC (SEQ ID No. 35)

EXAMPLE 6

Expression of NFPs in *E.coli*

To prepare a DNA construct for novel fluorescent protein expression, two primers were synthesized for each cDNA: a 5'-directed "downstream" primer with the annealing site located in the 3'-UTR of the cDNA and a 3'-directed "upstream" primer corresponding to the site of translation start site (not including the first ATG codon) (Table 6). Primers with SEQ ID Nos. 47 and 48 were the primers used to prepare the asFP600 DNA. Both primers had 5'-heels coding for a site for a restriction endonuclease; in addition, the upstream primer was designed so as to allow the cloning of the PCR product into the pQE30 vector (Qiagen) in such a way that resulted in the fusion of reading frames of the vector-encoded 6xHis-tag and NFP. The PCR was performed as follows: 1 µl of the 20-fold dilution of the amplified cDNA sample was added to a mixture containing 1x Advantage KlenTaq Polymerase Mix with buffer provided by the manufacturer (CLONTECH), 200 µM dNTPs, 0.2 µM of upstream primer and 0.2 µM of downstream primer, in a final total volume of 20 µl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of this amplification step was purified by phenol-chlorophorm extraction and ethanol precipitation and then cloned into pQE30 vector using restriction endonucleases corresponding to the primers' sequence according to standard protocols.

All plasmids were amplified in XL-1 blue *E. coli* and purified by plasmid DNA miniprep kits (CLONTECH). The recombinant clones were selected by colony color, and grown in 3 ml of LB medium

(supplemented with 100 μg/ml of ampicillin) at 37°C overnight. 100 μl of the overnight culture was transferred into 200 ml of fresh LB medium containing 100 μg/ml of ampicillin and grown at 37°C, 200 rpm up to OD_{600} 0.6-0.7. 1 mM IPTG was then added to the culture and incubation was allowed to proceed at 37°C for another 16 hours. The cells were harvested and recombinant protein, which incorporated 6x His tags on the N-terminus, was purified using TALON™ metal-affinity resin according to the manufacturer's protocol (CLONTECH).

TABLE 6

Primers Used to Obtain Full Coding Region of nFPs for Cloning into Expression Construct

Species	Upstream Primer	Downstream Primer
Anemonia majano	5' -acatggatccgctctttcaaaca agtttatc (SEQ ID No. 36) BamHI	5'-tagtactcgagcttattcgta tttcagtgaaatc (SEQ ID No. 37) XhoI
Clavularia sp.	L: 5'-acatggatccaacatttttttga gaaacg (SEQ ID No. 38) BamHI S: 5'-acatggatccaaagctctaacc accatg (SEQ ID No. 39) BamHI	5'-tagtactcgagcaacacaa accctcagacaa (SEQ ID No. 40) XhoI
Zoanthus sp.	5'- acatggatccgctcagtcaaag cacggt (SEQ ID No. 41) BamHI	5'-tagtactcgaggttggaactacat tcttatca (SEQ ID No. 42) XhoI
Discosoma sp. "red"	5'- acatggatccaggtcttccaagaat gttatc (SEQ ID No. 43) BamHI	5'-tagtactcgaggagccaagttc agcctta (SEQ ID No. 44) XhoI
Discosoma striata	5'- acatggatccagttggtccaagagtgtg (SEQ ID No. 45) BamHI	5'-tagcgagctctatcatgcctc gtcacct (SEQ ID No. 46) SacI
Anemonia sulcata	5'- acatggatccgcttcctttttaaagaagact (SEQ ID No. 47) BamHI	5'-tagtactcgagtccttgggagc ggcttg (SEQ ID No. 48) XhoI
Discosoma sp. "magenta"	5'- acatggatccagttgttccaagaatgtgat (SEQ ID No. 49) BamHI	5'-tagtactcgaggccattacg ctaatc (SEQ ID No. 50) XhoI
Discosoma sp. "green"	5'-acatggatccagtgcacttaaagaagaaatg (SEQ ID No. 51)	5'-tagtactcgagattcggtttaat gccttg (SEQ ID No. 52)

EXAMPLE 7

Novel Fluorescent Proteins and cDNAs Encoding the Proteins

One of the full-length cDNAs encoding novel fluorescent proteins is described herein (asFP600). The nucleic acid sequence and deduced amino acid sequence are SEQ ID Nos. 55 and 56, respectively. The spectral properties of asFP600 are listed in Table 7, and the emission and excitation spectrum for asFP600 is shown in Figures 2.

TABLE 7

Spectral Properties of the Isolated asFP600

Species:	Clavularia sp.	Max. Extinction Coefficient:	56,200
nFP Name:	asFP600	Quantum Yield	<0.01
Absorbance Max. (nm):	572	Relative Brightness:*	
Emission Max. (nm):	596		

*relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

EXAMPLE 8

Construction of asFP600 Mutant

One mutant of asFP600 was generated, Mut1. Mut1 has the nucleic acid sequence shown in SEQ ID No. 57 and deduced amino acid sequence shown in SEQ ID No. 58. Compared with wild type asFP600, Mut1 has the following substitutions: T to A at position 70 (numbering according to GFP) and A to S at position 148. Target substitution A148S was generated by means of site-specific mutagenesis using PCR with primers that carried the mutation. During this mutagenesis random substitution T70A was generated by introducing a wrong nucleotide in PCR. The substitution T70A is not necessary for fluorescence and practically does not affect the fluorescence. Figure 3

shows the emission and excitation spectrum for Mut1. Table 8 lists the spectral properties of Mut1.

TABLE 8

Spectral Properties of the Isolated Mut1

Species:	Anemonia Sulcata	Max. Extinction Coefficient:	15,500
nFP Name:	Mut1	Quantum Yield	0.05
Absorbance Max. (nm):	575	Relative Brightness:*	0.03
Emission Max. (nm):	595		

*relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

EXAMPLE 9

Construction and Functional Analysis of Vectors

Non-humanized mutant asFP600 (RNFP) DNA were amplified via PCR and reconstructed to EGFP-N1 backbone. This vector (pRNFP-N1) has the same multiple cloning sites as EGFP-N1.

Functional test of the generated vector was performed by transient transfection in 293 cells. 24 hours post transfection, expression of asFP600 was examined under fluorescent microscope. asFP600 showed good fluorescent intensity, however, the expression of asFP600 concentrated at the nucleus (Figure 4).

EXAMPLE 10

Generation of Cytosal Expressed asFP600

Since the nuclear localization of asFP600 limited some of the application of this protein as transcription reporter or pH sensor, cytosal expression of this protein would be needed for this purpose. A nuclear export sequence in humanized codon usage was fused to the N-terminus of asFP600, and placed into the EGFP-N1 vector, resulted in pNE-RNFP.

Functional test of NE-RNFP is performed by transient transfect the pNE-RNFP into 293 cells. 24 hours post transfection, expression of NE-RNFP is examined under fluorescence microscope. Red fluorescence was observed to be distributed in the cytosol but not in the nucleus (Figure 5).

EXAMPLE 11

Generation of Destabilized asFP600 Vectors as Transcription Reporters

Since asFP600 is very stable, it is necessary to generate destabilized versions of asFP600 in order to observe the rapid turnover of the protein. By using the same technology for destabilized EGFP, two destabilized NE-RNFP vectors were constructed by fusing mouse ODC degredation domain to the C-terminal of NE-RNFP. The d1 version of destabilized RNFP has three E to A mutations within MODC degradation domain comparing to d2 version, therefore result in a shorter half-life of the protein to which MODC degradation domain fused. Destabilized d1RNFP and d2RNFP were constructed in EGFP-N1 backbone.

EXAMPLE 12

Functional Analysis of Destabilized asFP600

d2 version of the none-humanized asFP600 was transiently transfected into 293 cells. One day after transfection, CHX was added to inhibit protein synthesis. 3 hours after treatment, cells were examined under fluorescent microscope. It showed that fluorescent intensity decreased ~50%.

EXAMPLE 13

Construction and Functional Test for Humanized Mut1

Humanized Mut1 was generated. The nucleic acid sequence of Mut1 is shown in SEQ ID No. 59. The humanized Mut1 was then placed into the pEGFP-N1 backbone. This vector has the same multiple cloning sites as pEGFP-N1. Construction of C1 and pEGFP is in the process.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will appreciate readily that the present invention is adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects and ends inherent therein. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein, are presently representative of preferred embodiments, are exemplary, and

are not intended as limitations on the scope of the invention. Changes to the methods and compounds, and other uses, will occur to those skilled in the art and are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A DNA sequence encoding a fluorescent protein selected from the group consisting of:

(a) an isolated DNA which encodes a fluorescent protein, wherein said DNA is from an organism from a Class Anthozoa and wherein said organism does not exhibit bioluminescence;

(b) an isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

(c) an isolated DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein.

2. The DNA sequence of claim 1, wherein said organism is from Sub-class Zoantharia.

3. The DNA sequence of claim 2, wherein said organism is from Order Actiniaria.

4. The DNA sequence of claim 3, wherein said organism is from Sub-order Endomyaria.

5. The DNA sequence of claim 4, wherein said organism is from Family Actiniidae.

6. The DNA sequence of claim 5, wherein said organism is from Genus Anemonia.

7. The DNA sequence of claim 6, wherein said organism is *Anemonia sulcata.*

8. A DNA sequence encoding a fluorescent protein selected from the group consisting of:

(a) an isolated DNA which encodes a fluorescent protein having a nucleotide sequence selected from the group consisting of SEQ ID Nos. 55, 57 and 59;

(b) an isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

(c) an isolated DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code, and which encodes a fluorescent protein.

9. The DNA sequence of claim 8, wherein said DNA encodes a fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58.

10. The DNA sequence of claim 8, wherein said DNA is selected from the group consisting of asFP600 and Mut1.

11. The DNA sequence of claim 8, wherein said DNA is selected from the group consisting of non-humanized and humanized DNA.

12. A vector capable of expressing the DNA sequence of claim 1 in a recombinant cell, wherein said vector comprising said DNA and regulatory elements necessary for expression of the DNA in the cell.

13. The vector of claim 12, wherein said DNA encodes a fluorescent protein having the amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58.

14. The vector of claim 12, wherein said vector is constructed by amplifying said DNA and then inserting the amplified DNA into EGFP-N1 backbone.

15. The vector of claim 14, wherein said DNA is selected from the group consisting of non-humanized and humanized DNA.

16. The vector of claim 12, wherein said vector is constructed by fusing different mouse ODC degradation domains to the C-terminal of said DNA and then inserting the fusion DNA into EGFP-N1 backbone.

17. The vector of claim 16, wherein said mouse ODC degradation domains are selected from the group consisting of d1, d2 and d376.

18. The vector of claim 16, wherein said DNA is selected from the group consisting of non-humanized and humanized DNA.

19. A host cell transfected with the vector of claim 12, wherein said cell is capable of expressing a fluorescent protein.

20. The host cell of claim 19, wherein said cell is selected from the group consisting of bacterial cells, mammalian cells, plant cell, yeast and insect cells.

21. The host cell of claim 20, wherein said mammalian cell is HEK 293 cell.

22. The host cell of claim 20, wherein said bacterial cell is an *E. coli* cell.

23. An isolated and purified fluorescent protein coded for by DNA selected from the group consisting of:

(a) an isolated DNA which encodes a fluorescent protein from an organism from Class Anthozoa, wherein said organism does not exhibit bioluminescence;

(b) an isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

(c) an isolated DNA differing from the isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein.

24. The isolated and purified fluorescent protein of claim 23, wherein said organism is from Sub-class Zoantharia.

25. The isolated and purified fluorescent protein of claim 24, wherein said organism is from Order Actiniaria.

26. The isolated and purified fluorescent protein of claim 25, wherein said organism is from Sub-order Endomyaria.

27. The isolated and purified fluorescent protein of claim 26, wherein said organism is from Family Actiniidae.

28. The isolated and purified fluorescent protein of claim 27, wherein said organism is from Genus Anemonia.

29. The isolated and purified fluorescent protein of claim 28, wherein said organism is *Anemonia sulcata*.

30. An isolated and purified fluorescent protein coded for by DNA selected from the group consisting of:

(a) isolated DNA which encodes a fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 56 and 58;

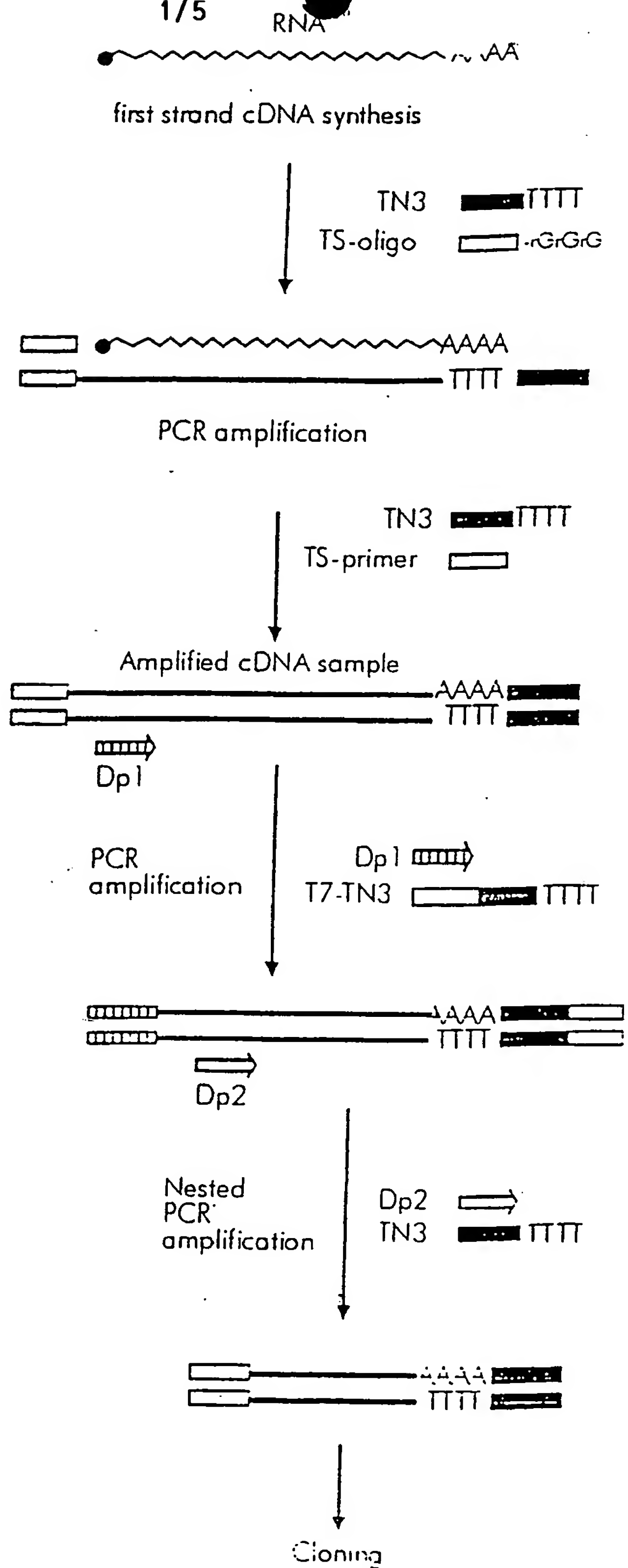
(b) isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a fluorescent protein; and

(c) isolated DNA differing from said isolated DNAs of (a) and (b) above in codon sequence due to degeneracy of the genetic code and which encodes a fluorescent protein.

31. The isolated and purifed fluorescent protein of claim 30, wherein said protein is asFP600.

32. An amino acid sequence which can be used as a basis for designing an oligonucleotide probe for identification of a DNA encoding a fluorescent protein by means of hybridizaton, wherein said sequence is selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12, 14.

33. The amino acid sequence of claim 32, wherein said oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15, 16.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AAAA
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amplification
Dp1
T7-TN3
TTTT
AAAA
TTTT
Dp2
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Cloning

Figure 1

max. extinction coefficient: 56,200
relative quantum yield: <0.01

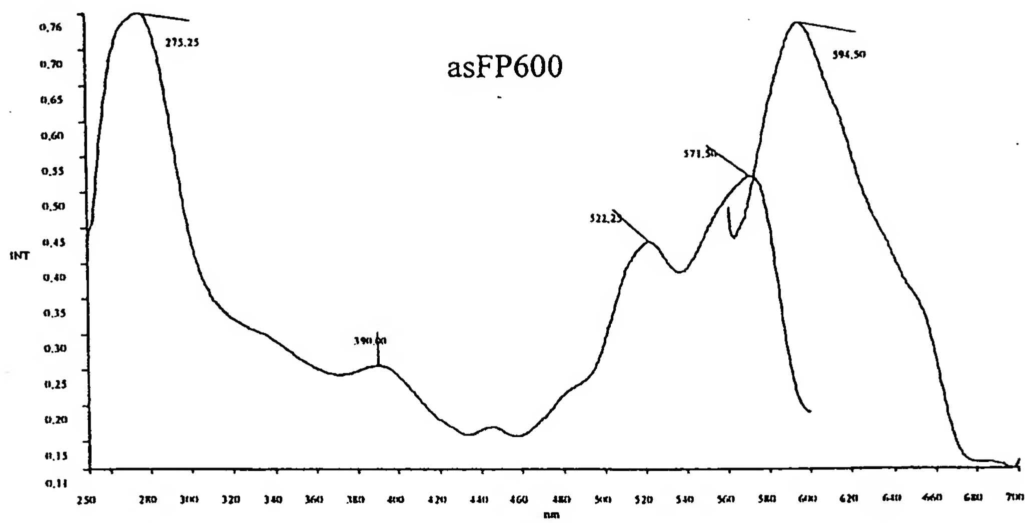
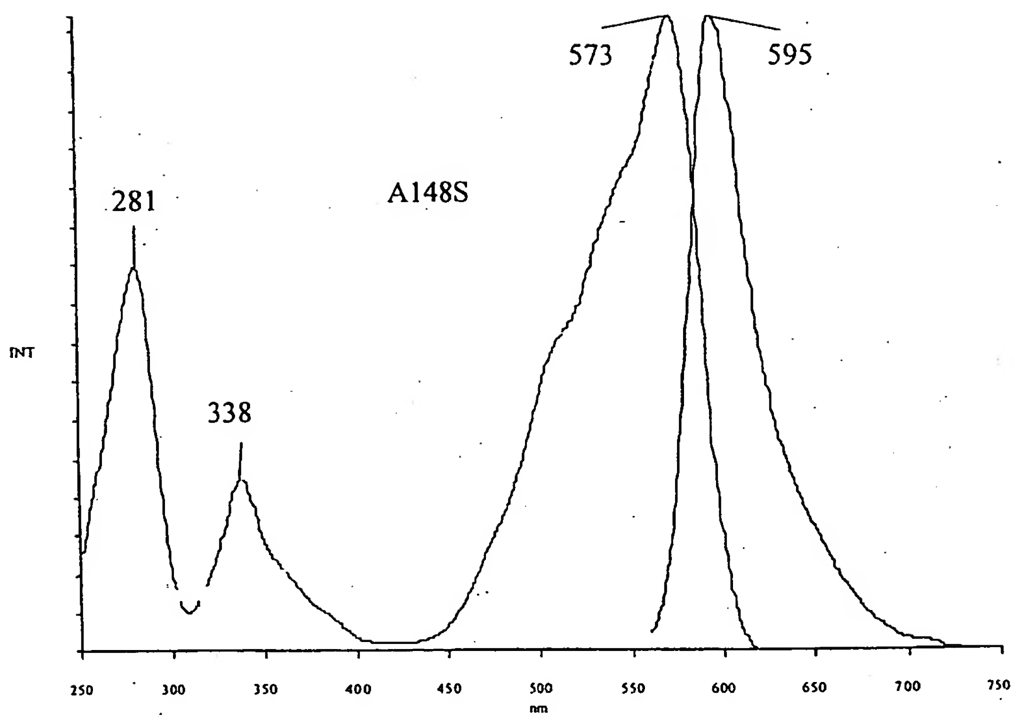


Figure 2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550
600
650
700
750
nm

Figure 3

Traci Yerby,11/22/99 9:46 AM -0000,

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d1wtNFP3.doc

Figure 4

Nuclear Exported NFP7 in 293 cells

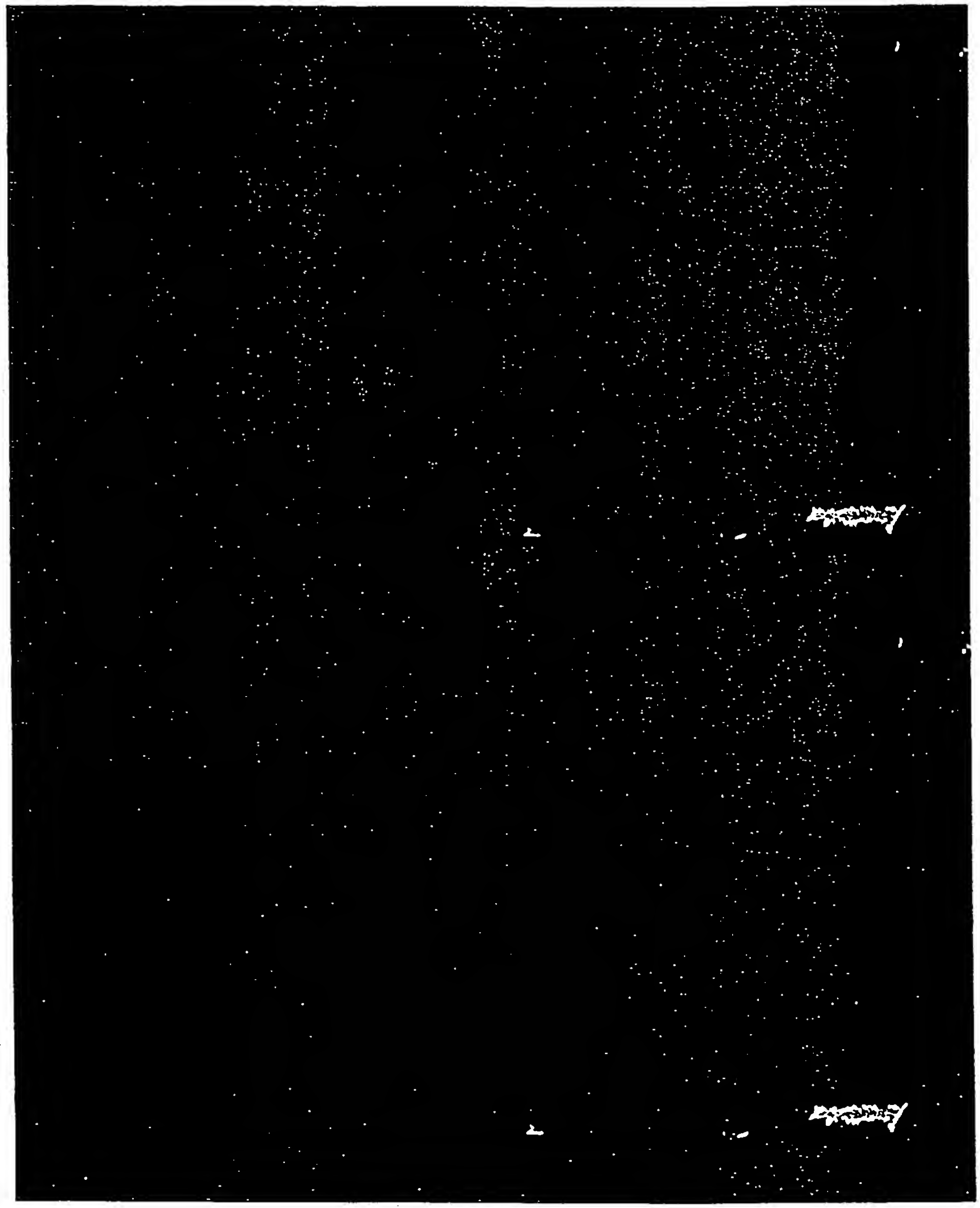

Figure 5

SEQUENCE LISTING

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Labas, Yulii A.
Matz, Mikhail V.
Fradkov, Arcady F.
Jiang, Xin
Duong, Tommy
Zhao, Xiaoning

<120> Fluorescent proteins from non-bioluminescent species of Class Anthozoa, genes encoding such proteins and uses thereof

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<210> 29
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<400> 29

ctgcacaacg ggtccat 17

<210> 30
<211> 20
<212> DNA
<213> artificial sequence
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<223> gene-specific primer used for 5'-RACE for *Anemonia sulcata*
<400> 30

cctctatctt catttcctgc 20

<210> 31
<211> 20
<212> DNA
<213> artificial sequence
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<221> primer_bind
<223> gene-specific primer used for 5'-RACE for *Anemonia sulcata*
<400> 31

tatcttcatt tcctgcgtac 20

<210> 32
<211> 19
<212> DNA
<213> artificial sequence
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<400> 32

ttcagcaccc catcacgag 19

<210> 33
<211> 19
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> gene-specific primer used for 5'-RACE for *Discosoma sp. "magenta"*
<400> 33

acgctcagag ctgggttcc 19

<210> 34
<211> 22
<212> DNA
<213> artificial sequence
<220>
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<223> gene-specific primer used for 5'-RACE for *Discosoma sp. "green"*
<400> 34

ccctcagcaa tccatcacgt tc 22

<210> 35

<211> 20
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<223> gene-specific primer used for 5'-RACE for *Discosoma sp. "green"*
<400> 35

attatctcag tggatggttc 20

<210> 36
<211> 31
<212> DNA
<213> artificial sequence
<220>
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<223> upstream primer used to obtain full coding region of nFPs from *Anemonia majano*
<400> 36

acatggatcc gctctttcaa acaagtttat c 31

<210> 37
<211> 34
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> downstream primer used to obtain full coding region of nFPs from *Anemonia majano*
<400> 37

tagtactcga gcttattcgt atttcagtga aatc 34

<210> 38
<211> 29
<212> DNA
<213> artificial sequence
<220>

<221> primer_bind
<223> upstream primer used to obtain full coding region of nFPs from *Clavularia sp.*
<400> 38

acatggatcc aacatttttt tgagaaacg 29

<210> 39
<211> 28
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> upstream primer used to obtain full coding region of nFPs from *Clavularia sp.*
<400> 39

acatggatcc aaagctctaa ccaccatg 28

<210> 40
<211> 31
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> downstream primer used to obtain full coding region of nFPs from *Clavularia sp.*
<400> 40

tagtactcga gcaacacaaa ccctcagaca a 31

<210> 41
<211> 28
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<223> upstream primer used to obtain full coding region of nFPs from *Zoanthus sp.*

<400> 41

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<210> 42
<211> 32
<212> DNA
<213> artificial sequence
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<221> primer_bind
<223> downstream primer used to obtain full coding region of nFPs from *Zoanthus sp.*
<400> 42

tagtactcga ggttggaact acattcttat ca 32

<210> 43
<211> 31
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> upstream primer used to obtain full coding region of nFPs from *Discosoma sp.* "red"
<400> 43

acatggatcc aggtcttcca agaatgttat c 31

<210> 44
<211> 29
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
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<400> 44

tagtactcga ggagccaagt tcagcctta 29

<210> 45
<211> 28
<212> DNA
<213> artificial sequence
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<400> 45

acatggatcc agttggtcca agagtgtg 28

<210> 46
<211> 28
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> downstream primer used to obtain full coding region of nFPs from *Discosoma striata*
<400> 46

tagcgagctc tatcatgcct cgtcacct 28

<210> 47
<211> 31
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<213> artificial sequence
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<400> 47

acatggatcc gcttcctttt taaagaagac t 31

<210> 48
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<212> DNA
<213> artificial sequence

<220>
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<400> 48

tagtactcga gtccttggga gcggcttg 28

<210> 49
<211> 30
<212> DNA
<213> artificial sequence
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<223> upstream primer used to obtain full coding region of nFPs from *Discosoma sp. "magenta"*
<400> 49

acatggatcc agttgttcca agaatgtgat 30

<210> 50
<211> 26
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> downstream primer used to obtain full coding region of nFPs from *Discosoma sp. "magenta"*
<400> 50

tagtactcga ggccattacg ctaatc 26

<210> 51
<211> 31
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> upstream primer used to obtain full coding region of nFPs from *Discosoma sp. "green"*

<400> 51

acatggatcc agtgcactta aagaagaaat g 31

<210> 52
<211> 29
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> downstream primer used to obtain full coding region of nFPs from *Discosoma sp.* *"green"*
<400> 52

tagtactcga gattcggttt aatgccttg 29

<210> 53
<211> 33
<212> DNA
<213> artificial sequence
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<221> primer_bind
<223> TS-oligo used in cDNA synthesis and RACE
<400> 53

aagcagtggt atcaacgcag agtacgcrgr grg 33

<210> 54
<211> 238
<212> PRT
<213> *Aequorea victoria*
<220>
<223> amino acid sequence of GFP
<400> 54

Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
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Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser
20 25 30

Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys
35 40 45

Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu
50 55 60

Val Thr Thr Phe Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro
65 70 75

Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu
80 85 90

Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn
95 100 105

Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val
110 115 120

Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn
125 130 135

Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val
140 145 150

Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe
155 160 165

Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp
170 175 180

His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu
185 190 195

Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp
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Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr
215 220 225

Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
230 235

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<400> 55

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gaaaaggaga gggcaaccca tttgagggta cgcaggaaat gaagatagag 200
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tggagattgc ctcgtttaca aggtcaagat tcttggtaat aattttcctg 450
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gagatagttt atgaagttga cggtgtcctg cgtggacagt ctttgatggc 550
ccttaagtgc cctggtggtc gtcatctgac ttgccatctc catactactt 600
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gaagatcatc gcatcgagat aatggaggaa gttgagaaag gcaagtgcta 700
taaacagtac gaagcagcag tgggcaggta ctgtgatgct gctccatcca 750
agcttggaca taactaagat acaagccgct cccaaggaca ccaggctgtg 800
ttaccattag ctgttagttc aagtcctgca tatttttcaa ttttctgcat 850
gttaccgact ttttgtttga gtatcgaaca gcttttatac tttttgtaga 900
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tagat 955

<210> 56
<211> 232
<212> PRT
<213> *Anemonia sulcata*
<220>
<223> amino acid sequence of asFP600
<400> 56

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5 10 15
Glu Gly Thr Val Asn Gly His Tyr Phe Lys Cys Thr Gly Lys Gly
20 25 30
Glu Gly Asn Pro Phe Glu Gly Thr Gln Glu Met Lys Ile Glu Val
35 40 45
Ile Glu Gly Gly Pro Leu Pro Phe Ala Phe His Ile Leu Ser Thr
50 55 60
Ser Cys Met Tyr Gly Ser Lys Thr Phe Ile Lys Tyr Val Ser Gly
65 70 75

Ile Pro Asp Tyr Phe Lys Gln Ser Phe Pro Glu Gly Phe Thr Trp
80 85 90

Glu Arg Thr Thr Thr Tyr Glu Asp Gly Gly Phe Leu Thr Ala His
95 100 105

Gln Asp Thr Ser Leu Asp Gly Asp Cys Leu Val Tyr Lys Val Lys
110 115 120

Ile Leu Gly Asn Asn Phe Pro Ala Asp Gly Pro Val Met Gln Asn
125 130 135

Lys Ala Gly Arg Trp Glu Pro Ala Thr Glu Ile Val Tyr Glu Val
140 145 150

Asp Gly Val Leu Arg Gly Gln Ser Leu Met Ala Leu Lys Cys Pro
155 160 165

Gly Gly Arg His Leu Thr Cys His Leu His Thr Thr Tyr Arg Ser
170 175 180

Lys Lys Pro Ala Ser Ala Leu Lys Met Pro Gly Phe His Phe Glu
185 190 195

Asp His Arg Ile Glu Ile Met Glu Glu Val Glu Lys Gly Lys Cys
200 205 210

Tyr Lys Gln Tyr Glu Ala Ala Val Gly Arg Tyr Cys Asp Ala Ala
215 220 225

Pro Ser Lys Leu Gly His Asn
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<210> 57
<211> 696
<212> DNA
<213> artificial sequence
<220>
<223> nucleic acid sequence of Mut1
<400> 57

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ccatttgcct tccacatttt gtcaacgagt tgtatgtacg gtagtaaggc 200
cttcatcaag tatgtgtcag gaattcctga ctacttcaag cagtctttcc 250
ctgaaggttt tacttgggaa agaaccacaa cctacgagga tggaggcttt 300
cttacagctc atcaggacac aagcctagat ggagattgcc tcgtttacaa 350

ggtcaagatt cttggtaata attttcctgc tgatggcccc gtgatgcaga 400
acaaagcagg aagatgggag ccatccaccg agatagttta tgaagttgac 450
ggtgtcctgc gtggacagtc tttgatggcc cttaagtgcc ctggtggtcg 500
tcatctgact tgccatctcc atactactta caggtccaaa aaaccagcta 550
gtgccttgaa gatgccagga tttcattttg aagatcatcg catcgagata 600
atggaggaag ttgagaaagg caagtgctat aaacagtacg aagcagcagt 650
gggcaggtac tgtgatgctg ctccatccaa gcttggacat aactaa 696

<210> 58
<211> 231
<212> PRT
<213> artificial sequence
<220>
<223> deduced amino acid sequence of Mut1
<400> 58

Ala Ser Phe Leu Lys Lys Thr Met Pro Phe Lys Thr Thr Ile Glu
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20 25 30
Gly Asn Pro Phe Glu Gly Thr Gln Glu Met Lys Ile Glu Val Ile
35 40 45
Glu Gly Gly Pro Leu Pro Phe Ala Phe His Ile Leu Ser Thr Ser
50 55 60
Cys Met Tyr Gly Ser Lys Ala Phe Ile Lys Tyr Val Ser Gly Ile
65 70 75
Pro Asp Tyr Phe Lys Gln Ser Phe Pro Glu Gly Phe Thr Trp Glu
80 85 90
Arg Thr Thr Thr Tyr Glu Asp Gly Gly Phe Leu Thr Ala His Gln
95 100 105
Asp Thr Ser Leu Asp Gly Asp Cys Leu Val Tyr Lys Val Lys Ile
110 115 120
Leu Gly Asn Asn Phe Pro Ala Asp Gly Pro Val Met Gln Asn Lys
125 130 135
Ala Gly Arg Trp Glu Pro Ser Thr Glu Ile Val Tyr Glu Val Asp
140 145 150
Gly Val Leu Arg Gly Gln Ser Leu Met Ala Leu Lys Cys Pro Gly
155 160 165

Gly Arg His Leu Thr Cys His Leu His Thr Thr Tyr Arg Ser Lys
170 175 180
Lys Pro Ala Ser Ala Leu Lys Met Pro Gly Phe His Phe Glu Asp
185 190 195
His Arg Ile Glu Ile Met Glu Glu Val Glu Lys Gly Lys Cys Tyr
200 205 210
Lys Gln Tyr Glu Ala Ala Val Gly Arg Tyr Cys Asp Ala Ala Pro
215 220 225
Ser Lys Leu Gly His Asn
230

<210> 59
<211> 696
<212> DNA
<213> artificial sequence
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<223> nucleic acid sequence of humanized Mut1
<400> 59

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tcgagggcac ccaggagatg aagatcgagg tgatcgaggg cggccccctg 150
cccttcgcct tccacatcct gtccacctcc tgcatgtacg gctccaaggc 200
cttcatcaag tacgtgtccg gcatccccga ctacttcaag cagtccttcc 250
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29300

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 14/435; C12N 1/00, 1/15, 1/21, 5/10, 15/12, 15/63
US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 252.3, 252.33, 325, 410, 254.11, 348, 369, 69.1; 530/350, 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	MATZ et al. Fluorescent proteins from nonbioluminescent Anthozoa species. Nature Biotechnology. October 1999, Volume 17, No. 10, pages 969-973, see entire document.	1-33
X, P	DE 197 18 640 A1 (WIEDENMANN) 22 July 1999 (22.07.99), see entire document.	23-31
A	US 5,491,084 A (CHALIFE et al) 13 February 1996 (13.02.96).	23-31
X	ANDERLUH et al. Cloning, sequencing , and expression of equinatoxin II. Biochemical and Biophysical Research Communications. 1996, Volume 220, No. 2, pages 437-442, see entire document.	1-5, 8, 12,19-27, 30

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 MARCH 2000	18 APR 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Terry J. Dey for GABRIELE ELISABETH BUGAISKY
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X L	MACEK et al. Intrinsic tryptophan fluorescence of equinatoxin II, a pore-forming polypeptide from the sea anemone Actinia equina L, monitors its interaction with lipid membranes. European Journal of Biochemistry. 1995, Volume 234, pages 329-335, entire document. Cited as "L" document because it establishes fluorescence of equinatoxin II.	23-27, 30 1-5, 8, 12, 19-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29300

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 8-11, 13, 30-33
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Since the sequence diskette (CRF) submitted by applicant is defective, a sequence search could not be performed. Accordingly, claims 8-11, 13 and 30-33 were searched only in-part, based on a word search.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/320.1, 252.3, 252.33, 324, 410, 254.11, 348, 369, 69.1; 530/350; 536/23.5

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Dialog files 155, 5, 434, 34, 358,28,44, 77 (Medline, Biosis, Scisearch, Derwent Biotech Abs., Oceanic Abs., Aquatic & Fish Abs., Dissertation Abs. Online, Conference Papers Index); STN-CAS files registry, CAPLUS; WEST files USPT, Derwent WPI
search terms: fluoresc?, bioluminesc?, protein?, polypeptide?, gene#, anthozo?, actiniar?, actiniid?, sulcata, coral?, cnidar?, anemon?, asFP600, masflkktm/sqsp, vngh/sqep, gegeg/sqep, gegng/sqep, gmnfp/sqep, gvnfp/sqep, gpvn/sqep